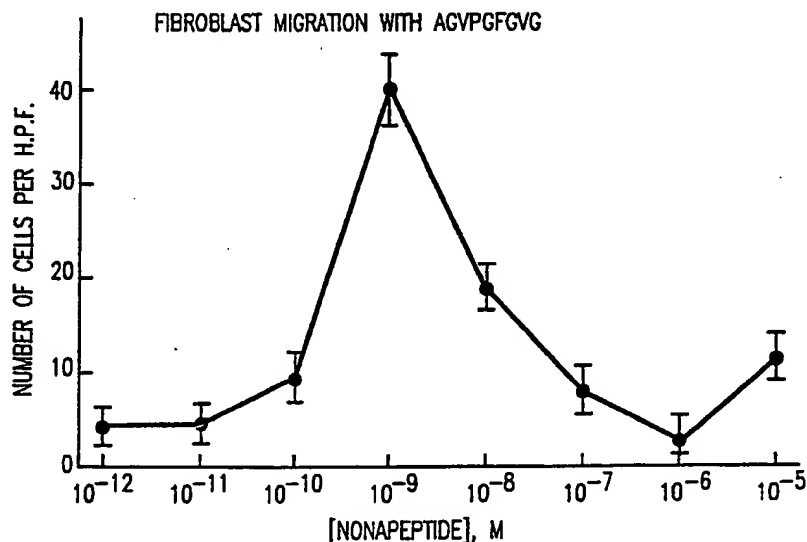




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(54) Title: STIMULATION OF CHEMOTAXIS BY CHEMOTACTIC PEPTIDES



(57) Abstract

A method of stimulating chemotaxis toward a prosthetic device is disclosed, which method comprises incorporating a chemotactic peptide of the formula $B^1-X-(AGVPGLGVG)_n-(AGVPGFGVG)_m-Y-B^2$, wherein B^1 is H or a biocompatible N-terminal group; B^2 is OH, OB^3 where B^3 is a non-toxic metal ion, or a biocompatible C-terminal group; X is GVPGFGVG, GVPGLGVG, VPGFGVG, VPGLGVG, PGFGVG, PGLGVG, GFGVG, GLGVG, FGVG, LGVG, GVG, VG, G or a covalent bond; Y is AGVPGFGV, AGVPGLGV, AGVPGFG, AGVPGLG, AGVPGF, AGVPGL, AGVPG, AGVP, AGV, AG, A or a covalent bond; n is an integer from 0 to 50; m is an integer from 0 to 50; with the proviso that when both n and m are 0, X and Y are selected so that the chemotactic peptide has at least 3 amino acid residues in the X and Y positions combined; into a surface of a prosthetic device.

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Description

Stimulation of Chemotaxis by Chemotactic
Peptides

Technical Field

This work was supported in part by grants from the
5 National Institutes of Health and the Government has
certain rights in the invention as a result of this
support.

This application is a continuation-in-part of U.S.
Serial No. 07/013,343, filed February 11, 1987, which
10 is a continuation of U.S. Serial No. 06/793,225, filed
October 31, 1985, now U.S. Patent 4,693,718.

Background Art

This invention relates to stimulation of
chemotaxis, particularly in relation to prosthetic
15 devices.

Disclosure of the Invention

Replacement of a blood vessel by a prosthetic
device is an important and common practice in modern
vascular surgery. Although some use is made of veins
20 or arteries taken from other portions of a patient's
body, most of such prosthetic devices are prepared from
artificial materials that can be prepared in a variety
of sizes and stored in a sterile state ready for use.

There are several essential properties of

cardiovascular prosthetic materials, among which are the following:

1. Retardation of thrombosis and thromboembolism (antithrombogenic);
- 5 2. Minimal harm to blood cells and minimal blood cell adhesion;
3. Long life as prosthetic inserts: and
4. High compliance with the physical and chemical properties of natural blood vessel such as similar
10 elastic modulus and tensile strength.

Another useful property would be a chemotactic response that induced rapid endothelialization and invasion of connective tissue cells for vascular wall reconstruction in a manner such that the prosthesis
15 would be slowly replaced by and/or integrated into newly synthesized internal elastic lamina. None of the materials presently being used can fulfill all of these requirements.

The most commonly used fabric for blood vessel
20 prosthesis is made from Dacron (Trademark, DuPont), a synthetic polyester fiber made from polyethylene terephthalate. Dacron has been used in several weaves and in combination with other materials. An example of a frequently used material is the DeBakey Elastic Dacron
25 fabric manufactured by USCI, a division of C.R. Bard, Inc. (Cat. No. 007830). Other commonly used materials are felted polyurethane and polytetrafluorethylene (Berkowitz et al, Surgery, 72, 221 (1972); Wagner et al, J. Surg. Res., 1, 53 (1956); Goldfarb et al,

Trans. Am. Soc. Art. Int. Org., XXIII, 268 (1977)). No chemotactic substance is normally used with these materials.

Another recent development in prosthetic devices is artificial skin of the type disclosed in Yannas and Burke, J. Biomed. Mat. Res., 14, 65-81 (1980). The artificial skin is a collagen/glycosaminoglycan (GAG) composite and had been successfully tested as full-thickness skin wound replacements. Such membranes have effectively protected wounds from infection and fluid loss for long periods of time without rejection and without requiring change or other invasive manipulation. Appropriately designed artificial skin of this type has retarded wound contraction, and the artificial skin has been replaced, at least in part, by newly synthesized connective tissue. Additional disclosure of this artificial skin is found in Yannas et al, ibid, 107-131 (1980), and Dagalakis et al, ibid, 511-528 (1980). No synthetic chemotactic substance is normally used with these materials.

One chemotactic material that might be useful in enhancing invasion of fibroblasts into such prosthetic devices is platelet-derived growth factor (PDGF), a potent fibroblast chemo-attractant. Unfortunately, PDGF cannot be synthesized and must be obtained from platelets, making the utilization of such a material on a wide scale impractical.

Recently, a chemotactic peptide has been identified in tropoelastin and is described in U.S. Patent 4,605,413. This material is a chemotactic peptide having a 6-amino-acid repeating unit of formula APGVGV and its active permutation VGVAPG, in which

A represents alanine, P represents proline, G represents glycine, and V represents valine. Although this material readily produces chemotaxis and is a natural component of the human body, therefore making
5 it particularly suitable for use in vivo, room remains for additional improvements in the field of chemotactic stimulation, for example in cell specificity and sensitivity.

Accordingly, there remains a need for an
10 artificial and easily synthesized chemotactic material capable of attracting fibroblasts and endothelial cells into prosthetic devices and thereby enhancing the incorporation of such devices into the regenerating natural tissue.

15 Best Mode for Carrying Out the Invention

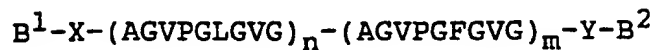
Accordingly, it is an object of this invention to provide a synthetic material having chemotactic properties towards cells such as fibroblasts and endothelial cells.

20 It is a further object of this invention to provide a prosthetic device which is readily incorporated into regenerating tissue, such as skin or blood vessel walls.

It is still another object of this invention to
25 provide a chemotactic material having stimulating activity to a greater extent than was previously available.

These and other objects of the invention as will hereinafter become more readily apparent have been
30 accomplished by providing a method of stimulating

chemotaxis, which comprises: incorporating a chemotactic peptide of the formula



- wherein A is a peptide-forming residue of L-alanine;
- 5 P is a peptide-forming residue of L-proline;
- G is a peptide-forming residue of glycine;
- V is a peptide-forming residue of L-valine;
- F is a peptide-forming residue of L-phenylalanine;
- 10 L is a peptide-forming residue of L-leucine;
- B^1 is H or a biocompatible N-terminal group;
- B^2 is OH, OB^3 where B^3 is a non-toxic metal ion, or a biocompatible C-terminal group:
- X is GVPGFGVG, GVPGLGVG, VPGFGVG, VPGLGVG,
- 15 PGFGVG, PGLGVG, GFGVG, GLGVG, FGVG, LGVG, GVG, VG, G or a covalent bond;
- Y is AGVPGFGV, AGVPGLGV, AGVPGFG, AGVPGLG, AGVPGF, AGVPGL, AGVPG, AGVP, AGV, AG, A or a covalent bond;
- 20 n is an integer from 0 to 50;
- m is an integer from 0 to 50; with the proviso that when both n and m are 0, X and Y are selected so that the chemotactic peptide has at least 3 amino acid residues in the X and Y positions combined;
- 25 into a layer of a prosthetic device in an amount sufficient to increase chemotaxis towards said layer.

This invention also comprises chemotactic matrices and prosthetic devices prepared according to the method set forth above.

Brief Description of the Drawings

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1 is a graph of the chemotactic dose response of fibroblasts to AGVPGFGVG;

10 FIGURE 2 is a graph of the chemotactic dose response of fibroblasts to GFGVGAGVP;

FIGURE 3 is a graph of the chemotactic dose response of fibroblasts to human platelet-derived growth factor (comparison);

15 FIGURE 4 is a graph of the chemotactic dose response of fibroblasts to VGVAPG (comparison);

FIGURE 5 is a carbon-13 nuclear magnetic resonance spectra of the Phe and Leu containing nonapeptides at 25 MHz, 29°C in DMSO-d₆. a. H-GFGVGAGVP-OH b.
20 H-GLGVGAGVP-OH;

FIGURE 6 is a phase contrast image of a typical monolayer of bovine aortic endothelial cells, which exhibit a "cobblestone" distribution pattern. After incubation with Dil-Ac-LDL, cells showed a punctate
25 fluorescence distribution pattern, indicative of endosomal localization of the labeled LDL derivative internalized by cells via receptor-mediated endocytosis. All cells were positive in fluorescent labeling with respect to controls, with variability in

cell to cell staining density typically present.

FIGURE 7 shows bovine aortic endothelial cell migration response to the elastin repeat nonapeptides; GFGVGAGVP ___ and GLGVGAGVP --°--. Net fibronectin migration for ___ curve was 30 cells per h.p.f. and 46 cells per h.p.f. for --°-- curve. Background migration was 38 and 95 cells h.p.f. for the two nonapeptides respectively.

FIGURE 8 shows bovine aortic endothelial cell migration in response to the elastin repeat peptide VGVAPG. Net fibronectin migration was 28 cells per h.p.f. and background migration was 38 cells per h.p.f.

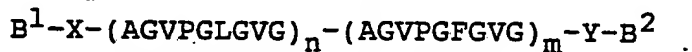
Description of the Preferred Embodiments

The present invention arose as the result of investigations into the structure of elastic fibers present in blood vessel walls and other elastic materials, such as ligaments, present in humans and animals. The central portion of the elastic fibers of vascular wall, skin, lung and ligament is derived from a single protein called tropoelastin. Polypeptide sequences of tropoelastin from vascular wall have been shown by Sandberg and colleagues to contain a repeat hexapeptide (Ala-Pro-Gly-Val-Gly-Val)_n, a repeat pentapeptide (Val-Pro-Gly-Val-Gly)_n, and a repeat tetrapeptide (Val-Pro-Gly-Gly)_n, where Ala, Pro, Val and Gly respectively represent alanine, proline, valine and glycine amino acid residues. (Peptide representations in this application conform to the standard practice of writing the NH₂-terminal amino acid residue at the left of the formula and the CO₂H-terminal amino acid residue at the right.) A high polymer of the hexapeptide has been synthesized,

whereby it forms cellophane-like sheets. The hexapeptide has therefore been thought to fill a structural role in the natural material.

However, recent investigations have indicated that
5 this hexapeptide and permutations of this sequence are chemotactic for fibroblasts which synthesize elastic fiber precursor protein in biological systems. As a result of this discovery and related investigations into the various permutations of the natural material,
10 U.S. Patent 4,605,413 discloses and claims a synthetic material based on the hexapeptide sequence.

Further investigations into tropoelastin have revealed the presence of a nonapeptide that repeats four times in a single continuous sequence.
15 Investigations into the property of materials based on this repeating unit had indicated that synthetic materials produced having this sequence are potent chemotactic agents having an activity even higher than synthetic materials based on the previously discovered
20 hexapeptide sequence. At their maximum activity, the nonapeptides are as active as platelet-derived growth factor (PDGF) at 30 ng/ml. The nonapeptides therefore are fully as potent as the previously discovered hexapeptides and achieve this activity at a comparable
25 or slightly lower concentration. It is expected that enhanced invasion of elastic-fiber-synthesizing fibroblasts and/or endothelial cells will occur when a prosthetic device, designed for incorporation into regenerating tissue, is treated by incorporating a
30 chemotactic peptide of the formula



wherein A is a peptide-forming residue of L-alanine;

P is a peptide-forming residue of L-proline;

G is a peptide-forming residue of glycine;
 V is a peptide-forming residue of L-valine;
 F is a peptide-forming residue of

L-phenylalanine;

5 L is a peptide-forming residue of L-leucine;

B¹ is H or a biocompatible N-terminal group;

B² is OH, OB³ where B³ is a non-toxic metal
 ion, or a biocompatible C-terminal group:

X is GVPGFGVG, GVPGLGVG, VPGFVG, VPGLGVG,
 10 PGFVG, PGLGVG, GFGVG, GLGVG, FGVG, LGVG, GVG, VG, G or
 a covalent bond;

Y is AGVPGFVG, AGVPGLGV, AGVPGFG, AGVPGLG,
 AGVPGF, AGVPGL, AGVPG, AGVP, AGV, AG, A or a covalent
 bond;

15 n is an integer from 0 to 50;

m is an integer from 0 to 50; with the proviso
 that when both n and m are 0, X and Y are selected so
 that the chemotactic peptide has at least 3 amino acid
 residues in the X and Y positions combined;

20 into a surface of the prosthetic layer. In this way
 the layer of the prosthetic device becomes the source
 of a concentration gradient of the chemotactic peptide.

Both the isolated nonamers, such as H-AGVPGFVG-
 OH, and polynona peptides have the chemotactic
 25 property. The nonapeptide H-GFGVGAGVP-OH has been
 shown to have essentially the same chemotactic activity
 as H-AGVPGFVG-OH. Chemotactic activity is also
 expected for the other permutations; i.e., H-GVPGFGVGA-
 OH, H-VPGFVGAG-OH, H-PGFGVGAGV-OH, H-FGVGAGVPG-OH,
 30 H-GVGAGVPGF-OH, H-VGAGVPGFG-OH, and H-GAGVPGFVG-OH.

When a polynona peptide is present, the compound
 (perhaps in the form of fragments derived therefrom by
in vivo enzymatic action) is chemotactic regardless of
 the value of n or m. However, for ease of handling,

values of n and m combined of no more than 100 are preferred since higher molecular weight compounds have limited solubility and are difficult to handle.

Preferred values of n and m are each from 1 to 10, with 5 values of about 5 being most preferred.

Preferably, B^1 and B^2 are H and OH, respectively. It can be seen from the above formula, that the peptides of the present invention can be made up of a repeating nonapeptide associated with n in the above formula or a repeating peptide associated with m in the above formula. When n is 0 and m is 1 or greater, the resulting compounds are the same as those in U.S. Serial No. 793,225, now U.S. Patent 4,693,718. When n and m are each 0, X and Y are selected so that the resulting chemotactic peptide has at least 3 amino acid residues in the X and Y positions. Still more preferably, when n and m are 0, X and Y are selected so that the resulting peptide has at least 5 amino acids in the X and Y positions combined. More preferably, the selected chemotactic peptide has from 5 to 40 amino acid residues. When the peptide has 5 amino acids, it is preferably GFGVG. Most preferably, the chemotactic peptide has 9 amino acid residues. It is also preferred that n is at least 1. It is also preferred that if m is greater than 0, n must be at least 1 or X or Y must be a residue containing a leucine residue. In a most preferred embodiment, n is 1 and m is 0.

It will be noted that polynonapeptides can be synthesized using any of the nonapeptide "monomers" listed above. Thus, polynonapeptides generally will have the structure $B^1-(\text{repeating unit})_n-B^2$ where B^1 and B^2 represent end groups which are discussed later. The

repeating unit can be any of the permutations of the nonamers listed above. In fact, if the chemotactic peptide is not synthesized from nonapeptide "monomers" but rather is synthesized by sequential addition of

5 amino acids to a growing peptide (such as in an automatic peptide synthesizer or by use of an artificial gene) the designation of a repeating unit is somewhat arbitrary. For example, the peptide H-GFGVGAGVPGFVGAGVPGFVGAGVPGF-OH can be considered to

10 consist of any of the following repeating units and end groups, among others: H-(GFGVGAGVP)₃-GF-OH, H-G-(FGVGAGVPG)₃-F-OH, H-GF-(GVGAGVPGF)₃-OH, H-GFG-(VGAGVPGFG)₂-VGAGVPGF-OH, H-GFGV-(GAGVPGFGV)₂-GAGVPGF-OH, or H-GFGVG-(AGVPGFGVG)₂-AGVPGF-OH.

15 Synthesis of the chemotactic peptide is straightforward and easily accomplished by a peptide chemist. The resulting peptides generally have the structure B¹-(repeating unit)_n(repeating unit)_m-B² where B¹ and B² represent any chemically compatible end group on the

20 amino and carboxyl ends of the molecule, respectively, and n and m are from 0 to about 50. When B¹ is H, B² is OH, n = 1 and m = 0, the compound is a nonapeptide. When n or m is greater than 1, the compound is a polynonapeptide (often referred to herein

25 as a polypeptide). It is possible that one or more amino acid residue or segment of amino acid residues not present in the normal polynonapeptide sequence may be interspersed within a polynonapeptide chain so long as the chemotactic character of the resulting molecule

30 is not completely disrupted. As clearly indicated by the formula and by the following discussion, the invention encompasses incorporation of a nonamer or polynonapeptide into a larger peptide chain in which B¹ and B² represent the remainder of the larger peptide

chain.

Other examples of terminal B¹ and B² end groups include portions of the repeating peptide units themselves with free amino or carboxylic acid groups or salts thereof, free amino or carboxylic acid groups or salts (especially alkali metal salts), and peptide or amino acid units that have retained a blocking group that was present during synthesis of the polypeptide or that have a biocompatible group added after formation of the polypeptide. Examples of blocking groups include t-butyloxycarbonyl, formyl, and acetyl for the amino end of the molecule and esters, such as methyl esters, as well as amides, such as the amides of ammonia and methyl amine, for the acid end of the molecule. The end groups are not critical and can be any organic or inorganic group that does not destroy the chemotactic properties of the polypeptide or confer bio-incompatibility to the molecule as a whole. The term biologically compatible as used in this application means that the component in question will not harm the organism in which it is implanted to such a degree that implantation is as harmful as or more harmful than the needed prosthetic device.

Methods of preparing polypeptide polymers have been disclosed in Rapaka and Urry, Int. J. Peptide Protein Res., 11, 97 (1978), Urry et al, Biochemistry, 13, 609 (1974), and Urry et al, J. Mol. Biol., 96, 101 (1975), which are herein incorporated by reference. The synthesis of these peptides is straightforward and can be easily modified to any of the peptides disclosed herein. The following summary, which is not to be considered limiting, is an example of the general method of synthesizing the polypeptides.

The first step in the formation of a polynona-peptide of the invention usually is synthesis of a nonapeptide monomer. Any of the classical methods of producing peptide molecules may be used in synthesizing
5 the building blocks of the polymers of the present invention. For example, synthesis can be carried out by classical solution techniques starting from the C-terminal amino acid as a benzyl (Bzl) ester p-tosylate. Each successive amino acid is then coupled
10 to the growing peptide chain by means of its water-soluble carbodiimide and 1-hydroxybenzotriazole. A typically used carbodiimide is 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (EDCI). During the coupling reaction the amino group is
15 protected. The protecting group is then removed after condensation has taken place. A suitable protecting group is tert-butyloxycarbonyl (Boc), which can easily be removed by trifluoroacetic acid.

The first product obtained in the synthesis of the
20 hexapeptide monomer is a protected nonapeptide, such as Boc-L-Ala-Gly-L-Val-L-Pro-Gly-L-Phe-Gly-L-Val-Gly-OBzl. This protected monomer is converted into the reactive monomer by, for example, replacement of the benzyl ester with the p-nitrophenyl ester, for example
25 by effectively exchanging with p-nitrophenyl trifluoroacetate, and removal of the Boc protecting group. The resulting reactive monomer is polymerized, in the presence of a base such as triethylamine as necessary, to give the polypeptide. A blocking group,
30 such as H-Val-OMe may be added at the conclusion of the polymerization reaction to convert the remaining reactive p-nitrophenyl esters to non-reactive terminal groups if desired.

Since all of the amino acids present in the

polypeptides of the invention have corresponding DNA codons, the polypeptides can also be produced by genetic engineering using synthetic genes containing codons that correspond to the desired amino acid sequence.

5 When a modified chemical structure is desired, as, for example, when chemical cross-linking between two chains of polynonapeptide or between a polynonapeptide chain and a peptide-forming part of the structure of a prosthetic device will be carried out, side-group-
10 blocked lysine or glutamic acid (or another amino acid with a protected side group capable of forming a cross-link after the protecting group is removed) may be utilized in place of one of the normal amino acids that is present in the polypeptide chain. A synthesis of a
15 chemically cross-linked polypentapeptide of similar structure is disclosed in U.S. Patent 4,187,852, which is herein incorporated by reference.

It is not necessary for the chemotactic peptide of the invention to be covalently attached to the surface
20 toward which chemotaxis is being stimulated. It is sufficient that the peptide be present at the surface or layer. Therefore, the phrase "incorporating into a surface or layer" as used in this application encompasses all methods of applying a chemotactic
25 peptide of this invention to a surface, whether that application results in chemical bonding or not. For example, solutions or suspensions containing the peptide can be painted on the surface of a prosthetic device or a device can be submerged in a solution of
30 the chemotactic peptide and be made to swell taking in the chemotactic peptide and then can be made to contract expelling the water but leaving the

chemotactic peptide within the matrix of the device.

It is also possible to form covalent bonds between the chemotactic peptide and the prosthetic device. For example, during the synthesis of a chemotactic peptide
5 as described above, various intermediates are produced which have reactive carboxy or amino terminals. Many of the prosthetic devices which are intended for incorporation into regenerating tissue are prepared from collagen or related materials and therefore
10 contain free amino acid functional groups, such as amino or carboxylic acid groups. Peptide bonds can easily be formed between such functional groups in the prosthetic device and reactive intermediates such as those described above.

15 The type of prosthetic device which can be used in conjunction with the present invention is not limited, since the chemotactic property is related to the peptide and not to the prosthetic device itself. It is preferred, however, that the prosthetic device be one
20 which is intended for incorporation into regenerating tissue, such as an artificial vein or artery or artificial skin. Publications which disclose various prosthetic devices useful for forming artificial skin or blood vessel walls are listed in the section of this
25 application entitled Background of the Invention, and these publications are herein incorporated by reference. Two particularly preferred embodiments of the present invention involve using the chemotactic polypeptide with a collagen/glycosaminoglycan composite
30 material as an artificial skin, as described in U.S. Patent 4,280,954, and with biocompatible artificial materials based on polypeptides as described in U.S. Patent 4,187,852: U.S. Patent Application Serial Number

308,091, filed October 2, 1981; and U.S. Patent Application 452,801, filed December 23, 1982, all of which are herein incorporated by reference. These are peptide-containing materials, and the chemotactic polypeptide may readily be attached by covalent bonding into such materials by the methods described above. However, as also previously indicated, covalent bonding is not necessary and indeed is not preferred since the chemotactic property is also exhibited when the chemotactic peptide is merely present on the surface or in the pores of a prosthetic material. Prosthetic devices having surfaces comprising other structural peptides are also preferred over prosthetic devices having other types of surfaces, although other types of surfaces, such as Dacron, and other synthetic fibers, are specifically included. Examples include natural materials such tendons or ligaments (for example, those transferred from one location to another within the same body) and synthetic or semi-synthetic materials. Semi-synthetic materials are those derived by manipulation of natural materials, such as collagen.

The amount of chemotactic peptide which is required for a particular prosthetic device can easily be determined by simple experimentation. Generally, quite low concentrations of the chemotactic peptide are required. For example, doping of a non-chemotactic surface to produce low concentrations of 0.1 nM to 100 nM of a chemotactic peptide at the surface will be sufficient. Generally, from 10^{-9} to 10^{-3} millimoles of nonamer or repeating unit of a polynona-peptide per 100 cm^2 of surface is sufficient for this purpose. It is preferred to produce a concentration of the chemotactic nonamer of from 10^{-10} to 10^{-6} M, preferably 10^{-9} to 10^{-7} M at the responsive cell.

Alternatively or additionally, a 2-component synthetic bioelastomer comprising the chemotactic peptide of this invention and the elastic polypentapeptide or polytetrapeptide of U.S. Patent 5 4,187,852 would act as a chemotactic elastic biopolymer which could be utilized for a variety of purposes. It is also possible to use the chemotactic peptide of this invention in a system involving natural crosslinking of synthetic bioelastomers, as is described in U.S. Patent 10 Application Serial Number 533,524, now Patent No. 4,589,882, which is herein incorporated by reference. That application discloses bioelastomers which are enzymatically cross-linked by lysyl oxidase.

The above disclosure generally describes the 15 present invention. A more complete understanding can be obtained by reference to the following specific examples, which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

20 Example 1

The chemotactic response of fibroblasts to two nonapeptides of the invention was measured using the techniques described below.

Materials and Methods

25 Source of Chemicals and Materials

Dulbecco's Modified Eagle Medium (DMEM), nonessential amino acids, L-glutamine, fetal bovine serum, penicillin-streptomycin solution: GIBCO, Chagrin, Ohio. Trypsin 1-300: ICN, Cleveland, Ohio. 30 Ethylene diaminetetraacetic acid (EDTA) - sodium salt

and gelatin: Sigma, St. Louis, Mo. Human serum albumin (HSA): American National Red Cross, Washington, D.C. Hematoxylin: Harleco, Gibbstown, NJ. Platelet-derived growth factor (PDGF):
5 Calbiochem., San Diego, CA. Polycarbonate membranes: Nucleopore Corp., Pleasanton, CA. Cellulose nitrate membranes: Millipore Corporation, Bedford, MA.

Cells

Passage-two fetal bovine ligamentum nuchae
10 fibroblast cell cultures were grown to confluency in 75 cm² plastic tissue culture flasks with Dulbecco's Modified Eagle (DME) medium containing 10% fetal calf serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, penicillin (100 U/ml), streptomycin
15 (100 µg/ml). These passage-two cell cultures were obtained from passage-one cell cultures provided by R. M. Senior and R. B. Mecham (Washington University, St. Louis, MO). Ligamentum nuchae explants were obtained from fetal calves greater than 180 days in gestational
20 age. Because of the possibility of platelet derived factors being present in the fetal calf serum, the cells were harvested after a 48 hour fast. At one to two days past confluency, the cells were dispersed by trypsin (0.025% trypsin, 0.1% EDTA in phosphate
25 buffered saline, pH 7.4) and washed two times in a second DME medium containing 2 mM glutamine, 0.1 mM nonessential amino acids and 1 mg/ml human serum albumin. Cell concentration was determined with a hemocytometer and adjusted to a final concentration of
30 1.5×10^5 cells/ml. The cells were used as is for the chemotaxis assay.

Chemotaxis Assay

The chemotaxis experiment used a 30 hole multi-blindwell plate as an adaptation of the modified Boyden chamber. The upper and lower compartments were
5 separated by an 8 μ m polycarbonate membrane superimposed on a 0.45 μ m cellulose nitrate membrane. The 8 μ m membrane was pretreated with 5% gelatin to enhance fibroblast attachment. The lower compartment contained 0.24 ml of the second DME medium plus/minus
10 chemotactic peptides while the upper one had 0.37 ml of the second DME medium with fibroblasts. In the checkerboard assay, peptide was also added to the upper compartment. The filled plate was placed in a humidified incubator at 37°C with 5% CO₂-air for 6
15 hours, after which the cell suspension was aspirated and the membranes were recovered, fixed in ethanol, stained in Harris' Alum hematoxylin, dehydrated in a graded series of propanol and cleared in xylene. Quantitation of cell locomotion to the area between the
20 two membranes was done with a light microscope, fitted with a bright field objective and eye piece grid, at 400 x. There were 3 membrane pairs per experimental condition and 5 randomly chosen fields were examined per pair. Every experiment had a negative control,
25 i.e. medium alone, and a positive control, i.e. platelet-derived growth factor, PDGF, in the lower compartment.

Number of cells per high power field are expressed in FIGURES 1 through 3 and in Tables I and II as the
30 net number of cells that migrated through the polycarbonate membrane. The number of cells moving with medium alone in the lower compartment was subtracted from the number of cells moving in response

to one of the peptides.

Table I

Checkboard Analysis of AGVPGFGVG with Fibroblasts

		Peptide concentration above filters (M)			
		0	10 ⁻¹⁰	10 ⁻⁹	10 ⁻⁸
Peptide con. below filters (M)	0	(10)0 ± 1.3	-2 ± 1.1	-2 ± 0.5	-3 ± 0.9
	10 ⁻¹⁰	6 ± 1.3	-3 ± 1.0	-4 ± 0.8	2 ± 1.8
	10 ⁻⁹	25 ± 1.6	3 ± 1.7	-4 ± 0.6	-2 ± 1.1
	10 ⁻⁸	9 ± 2.3	5 ± 1.6	5 ± 1.2	-2 ± 1.1

Results are expressed as means ± S.E.M. where n = 15. Positive control, PDGF at 60 µg/ml = 21.

Table II

Checkboard Analysis of GFGVGAGVP with Fibroblasts

		Peptide concentration above filters (M)			
		0	10 ⁻¹⁰	10 ⁻⁹	10 ⁻⁸
Peptide con. below filters (M)	0	(9)0 ± 1.0	1 ± 1.0	3 ± 1.2	-2 ± 1.4
	10 ⁻¹⁰	13 ± 1.6	-1 ± 0.8	-3 ± 0.7	-1 ± 0.8
	10 ⁻⁹	45 ± 3.4	7 ± 1.8	-2 ± 1.0	-1 ± 1.1
	10 ⁻⁸	21 ± 1.5	12 ± 1.6	7 ± 1.5	0 ± 1.50

Results are expressed as mean ± S.E.M. where n = 15. Positive control, PDGF at 30 µg/ml = 59.

Peptide Synthesis

Elemental analyses were carried out by MicAnal, Tuscon Az. All amino acids are of L-configuration except for glycine. Tertiary-butyloxcarbonyl (Boc) amino acids and amino acid benzyl esters (Bzl) were purchased from Bachem, Inc., Torrance, CA. Thin-layer chromatography was performed on silica gel plates obtained from Whatman, Inc., NJ and mentioned as R_f values in different solvent systems. R_f^1 chloroform (C), methanol (M), acetic acid (A), 95:5:3; R_f^2 CMA (85:15:3); R_f^3 CMA (75:25:3); R_f^4 ethyl acetate, acetic acid, ethanol (90:10:10). The hexapeptide was synthesized and purified as outlined in Senior et al., J. Cell Biol. 99: 870-874 (1984). The synthesis of the nonapeptides is carried out by solution methods and is presented in Schemes 1 and 2.

CF₃CO₂H·H-Gly-Val-Gly-OBzl (II): Boc-Gly-Val-Gly-OBzl (I) (10.0 g, 23.72 mmol) was stirred with 100 ml of trifluoroacetic acid (TFA) for one hour and solvent removed under reduced pressure. The residue was
5 trituated with ether, filtered, washed with ether and dried, to give 9.4 g (yield: 91%) of the deblocked peptide.

Boc-Phe-Gly-Val-Gly-OBzl (III): Boc-Phe-OH (9.16 g, 34.54 mmol) was dissolved in 100 ml of dimethyl-
10 formamide (DMF), cooled to 0°C and N-methylmorpholine (NMM) (3.83 ml) added. The solution was cooled to -15°C and isobutyl chloroformate (IBCF) (3.96 ml, 30 mmol) was added slowly under stirring while maintaining the temperature at -15 ± 1°C. After stirring at this
15 temperature for 15 minutes, a precooled solution of II (9.4 g, 21.59 mmol) and NMM (2.4 ml) in DMF (40 ml) was added and stirring continued for two hours at ice-bath temperature. A saturated solution of KHCO₃ was added to bring the pH to 8.0 and stirred for an additional 30
20 minutes. The reaction mixture was poured into a cold 90% saturated NaCl solution (1000 ml) and the precipitate obtained was filtered, washed with satd. NaCl, H₂O and dried. The peptide was crystallized from ethyl acetate to give 10 g of III (yield: 81.5%). R_f^2
25 0.83 Anal. Calcd. for C₃₀H₄₀N₄O₇: C 63.35, H 7.09, N 9.85%. Found: C 62.96, H 7.37, N 9.86%.

Boc-Gly-Phe-Gly-Val-Gly-OBzl (IV): III (8.0 g, 14 mmol) was deblocked as described for II and coupled with Boc-Gly-OH by the excess mixed anhydride method and
30 worked up as described under III to obtain the title compound in 98.6% yield, R_f^2 0.75. Anal. Calcd. for C₃₂H₄₃N₅O₈: C 61.42, H 6.92, N 11.19%. Found: C 61.12, H 7.21, N 11.19%.

Boc-Val-Pro-Gly-Phe-Gly-Val-Gly-OBzl (VI): Boc-Val-Pro-OH (V) (29) (1.89 g, 6.02 mmol) and 1-hydroxybenzotriazole (HOBt) (0.92 g, 6.02 mmol) were taken in DMF (20 ml), cooled with ice-salt freezing mixture and
5 reacted with 1(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (1.15 g, 6.02 mmol) for 15 min. IV was deblocked as described under II and an ice-cold solution of this deblocked peptide (3.5 g, 5.4 mmol), NMM (0.61 ml) in DMF (20 ml) was added to the
10 above activated acid component and stirred for two days at room temperature. Solvent was removed under reduced pressure. The residue was taken in chloroform and washed with water, 20% citric acid, water, satd. NaHCO_3 , water, dried over anhyd. MgSO_4 and solvent
15 removed under reduced pressure to give the desired peptide (3.4 g, yield: 75.7%), R_f^1 0.27, R_f^2 0.71 Anal. Calcd. for $\text{C}_{42}\text{H}_{59}\text{N}_7\text{O}_{10}$: C 61.36, H 7.23, N 11.93%. Found: C 61.25, H 7.66, N 12.08%.

Boc-Ala-Gly-OBzl (VII): This peptide was prepared by
20 the mixed anhydride method as described for the synthesis of III in 81% yield, R_f^1 0.24, R_f^4 0.92. Anal. Calcd. for $\text{C}_{17}\text{H}_{24}\text{N}_2\text{O}_5$: C 60.16, H 7.13, N 8.25%. Found: C 59.68, H 7.17, N 8.09%.

Boc-Ala-Gly-OH (VIII): VII (16.97 g, 50 mmol) was
25 taken in glacial acetic acid (170 ml) and hydrogenated overnight in the presence of 10% Pd/C (1.7 g) as catalyst at 40 psi. The catalyst was filtered with the aid of celite and solvent removed under reduced pressure. The residue was taken in satd. NaHCO_3
30 solution, extracted with EtOAc (3x), cooled, acidified to pH 2.0 and re-extracted with EtOAc (3x). The combined EtOAc extracts were washed with satd. NaCl,

and concentrated under reduced pressure. The residue was triturated with EtOAc: Pet. ether (1:1) and the precipitate obtained was filtered, washed with pet. ether and dried (10.9 g, yield: 87.5%), R_f^4 0.52. Anal
5 Calcd. for $C_{10}H_{18}N_2O_5 \cdot 1/2H_2O$: C 46.50, H 7.41, N 10.84%. Found: C 46.64, H 7.41, N 10.84%.

Boc-Ala-Gly-Val-Pro-Gly-Phe-Gly-Val-Gly-OBzl (IX):
VIII was coupled with the deblocked VI using EDCI/HOBt as described for the preparation of VI to give the
10 desired peptide in 84.8% yield. R_f^2 0.76, R_f^3 0.28. Anal. calcd. for $C_{47}H_{67}N_9O_{12}$: C 59.22, H 7.08, N 13.22%. Found C 58.73, H 7.55, N 13.14%.

Boc-Ala-Gly-Val-Pro-Gly-Phe-Gly-Val-Gly-OH (X): The nonapeptide benzylester (IX) was hydrogenated as
15 described under VIII to obtain the acid in quantitative yield. R_f^3 0.24. Anal. calcd. for $C_{40}H_{61}N_9O_{12} \cdot H_2O$: C 55.09, H 7.16, N 14.3%. Found: C 54.69, H 7.16, N 13.81%.

$HCO_2H \cdot H$ -Ala-Gly-Val-Pro-Gly-Phe-Gly-Val-Gly-OH (XI):
20 X (20 mg, 0.023 mmol) was stirred with 95-97% formic acid (0.6 ml) for 6 hours and concentrated under reduced pressure. The residue was taken in dist. water and lyophilized to obtain the product in quantitative yield. R_f^3 0.65. This completes synthesis of the first
25 permutation.

Boc-Ala-Gly-Val-Pro-OBzl (XIII): To obtain the second permutation, Boc-Val-Pro-OBzl (XII) was deblocked with TFA and coupled with VIII as described under VI to obtain the protected tetrapeptide in 67.4%
30 yield. R_f^2 0.80, R_f^4 0.80. Anal calc. for $C_{27}H_{40}N_4O_7$: C 60.54, H 7.52, N 10.46%. Found: C 60.80, H 7.53, N

10.56%.

Boc-Gly-Phe-Gly-Val-Gly-OH (XIV): IV was hydrogenated and worked up as described for VIII to obtain the product in quantitative yield. R_f^3 0.16. Anal. calcd. for $C_{25}H_{37}N_5O_8 \cdot H_2O$: C 54.23, H 7.10, N 12.65%. Found: C 54.14, H 6.93, N 12.21%.

Boc-Gly-Phe-Gly-Val-Gly-Ala-Gly-Val-Pro-OBzl (XV): After deblocking XIII with TFA, the salt was coupled with XIV using EDCI/HOBt and worked up as for the preparation VI. The title compound was obtained in 75.5% yield. R_f^2 0.57. Anal. calcd. for $C_{47}H_{67}N_9O_{12} \cdot 2H_2O$: C 57.06, H 7.23, N 12.74%. Found C 54.23, H 7.10, N 12.74%.

Boc-Gly-Phe-Gly-Val-Gly-Ala-Gly-Val-Pro-OH (XVI): The above peptide XV was hydrogenated as described for the preparation VIII to obtain the product in quantitative yield. R_f^3 0.22. Anal. calcd. for $C_{40}H_{61}N_9O_{12}$: C 55.66, H 7.12, N 14.60%. Found: C 55.86, H 7.23, N 14.14%.

$HCO_2H \cdot H$ -Gly-Phe-Gly-Val-Gly-Ala-Gly-Val-Pro-OH (XVII): The peptide XVI was treated with formic acid and worked up as described under XI. R_f^3 0.06. This completes the synthesis of the second permutation.

Results

Carbon-13 nuclear magnetic resonance spectra for the two nonapeptides demonstrated their purity. The elastin nonapeptide AGVPGFGVG (compound XI) and its permutation GFGVGAGVP are both chemoattractants for elastin synthesizing ligamentum nuchae fibroblasts.

FIGURE 1 shows the positive migration of fibroblasts in response to a concentration gradient ranging from 10^{-12} to 10^{-5} M nonapeptide. Maximal activity was at 10^{-9} M for AGVPGFGVG. Four such curves were run and all
5 peaked at 10^{-9} M. Its permutation GFGVGAGVP (compound XVII) was tested with a concentration range of 10^{-12} to 10^{-5} (FIGURE 2). Again the maximal response was at 10^{-9} M. Again, four experiments were completed and in all 10^{-9} M represented maximal activity. FIGURE 3
10 shows the response of the fibroblasts to human platelet derived growth factor at 0.3, 3, 10, 20, 30, 40, 50, 60, 70, 90, 300 and 3000 ng/ml. Maximum activity is at 30 ng/ml (1 nM). The hexapeptide, VGVA PG, of tropoelastin was chemotactic for fibroblasts with the
15 peak of activity at 10^{-8} M (FIGURE 4). Two such tests were done and both peaked at 10^{-8} M. The "checkerboard analysis" was performed with the two nonapeptides to identify the fibroblast migration as chemotaxis. Peptide was added to the lower compartment, to the
20 upper compartment (with the cells) or to both compartments to set up a positive, a negative or zero gradient, respectively. Tables I and II for AGVPGFGVG and GFGVGAGVP show that the cells preferentially migrated in response to a positive gradient and not to
25 a negative gradient or to no gradient. The experiments represented by the data in Tables I and II were repeated three times and two times, respectively. In all cases, the data followed the patterns seen in Tables I and II. The data demonstrate that the
30 peptides stimulated direct (chemotaxis) rather than random (chemokinesis) migration of fibroblasts and that the peptides are true chemoattractants. Relative to the positive control of 30 μ g/ml PDGF, the nonapeptides stimulated the same level of migration as PDGF.

Example 2Materials and Methods:Source of Chemicals and Materials

Modified Medium 199, fetal bovine serum trypsin-
5 EDTA and antibiotic antimycotic solution were purchased
from GIBCO, Chagrin, OH. The sources of the
polycarbonate and cellulose nitrate membranes were
Nucleopore Corp., Pleasanton, CA and Millipore Corp.,
Bedford, MA. The hematoxylin stain was from Harleco of
10 Gibbstown, N.J. and the Hemacolor stain was purchased
from American Scientific Products, Stone Mountain,
GA. Human plasma fibronectin was purchased from
Calbiochem, San Diego, CA.

Cell culture and cell preparation

15 Bovine thoracic aorta endothelial cells were
isolated by scraping with a scalpel blade. Harvested
cells were washed by centrifugation in Medium 199 with
Hank's salt solution containing penicillin-streptomycin
and 10% fetal bovine serum. After dissociation of cell
20 aggregates by extrusion through a 27 gauge needle,
cells were seeded in 25 cm² flasks at densities of
about 10⁴ cells/cm² and reached confluency in 3 to 4
days. Cells were grown in Medium 199, 10% fetal bovine
serum and 5% Ryan's growth supplement (Dr. U.S. Ryan,
25 U. Miami, FL). When cells reached confluency, they
were subcultured at a 1:3 split ratio, after removal
from culture dishes with a Costar cell scraper. Cells
were monitored for purity to specific fluorescent
staining of endothelial uptake by acetylated low
30 density lipoprotein (Stein and Stein, 1980, Netland et
al, 1985) labeled with 1,1'-dioctadecyl-1,3,3,3',3'-
tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL,

Biomedical Technologies, Stoughton, MA). All studies were performed on first to fifth passage cells. These early passage cells at one to two days past confluency were dissociated in 0.05% trypsin, 0.02% EDTA in Hanks
5 Balanced Salt Solution for 3 minutes at 37°C, centrifuged, washed 2 times and resuspended in Medium 199 without fetal calf serum. The trypsin reaction was stopped with soybean trypsin inhibitor at 1 mg/ml. Cell concentration was determined with a hemocytometer
10 and adjusted to a final concentration of 10^6 /ml.

Chemotaxis Assay

Chemotactic response was assayed with a 30 hole multi-blind-well plate as an adaptation of the modified Boyden chamber with the double membrane technique.
15 A collagen coated 8μ polycarbonate, polyvinyl pyrrolidone (PVP) free, membrane superimposed on a 0.45μ cellulose nitrate membrane separated the lower and upper compartments containing respectively 0.24 ml of test peptide dissolved in Medium 199 and 0.37 ml of
20 the cell suspension. The collagen pretreatment was intended to enhance cell attachment to the polycarbonate membrane. In the checkerboard assay, to distinguish between directed cell migration (chemotaxis) and random cell locomotion (chemokinesis),
25 the concentration gradient between the upper and lower chambers were abolished by adding the test peptide at various concentrations to the cell suspension. For all experiments medium alone in the bottom chamber served as the baseline control. Human plasma fibronectin at
30 $100\mu\text{g}/\text{ml}$ in the bottom was the positive control. The filled plate was incubated for 5 hours at 37°C in a humidified incubator with 5% CO_2 -air. The membrane pairs were recovered, fixed in ethanol, stained in

- Harris' alum hematoxylin, hydrated in a graded series of propanol and cleared in xylene. The membrane pairs were mounted on glass slides and cell locomotion quantitated in the volume between the two membranes
- 5 using bright field at 400X. Five randomly chosen fields were counted per membrane pair with there being 3 membrane pairs per experimental condition. The average of the 15 fields was calculated as was the standard error of the mean (S.E.M.) where n was 15.
- 10 Net cell migration was plotted as a function of peptide concentration with net migration being the average number of cells moving in response to the peptide minus the average number of cells moving in response to medium alone.
- 15 The chemotactic response was also assayed with the Neuroprobe (Bethesda, MD) micro blind-well chamber. The lower wells contained 30 μ l of Medium 199 + test peptide of fibronectin, and the upper wells 55 μ l of the cells suspended in Medium 199. A 8- μ m pore size.
- 20 PVP-free, rectangular, polycarbonate, gelatin and fibronectin treated, membrane separated the upper and lower chambers. The gelatin pretreated membrane was incubated in a PBS solution of 100 μ g/ml fibronectin at room temperature for 2 hours, air dried and used within
- 25 2 weeks. Once the cells were added to the assembled chamber, it was incubated at 37°C in 95% air - 5% CO₂ for 4 hours. The filter was removed, the top layer of nonmigrating cells scraped off and the bottom layer of migrating cells fixed in ethanol and stained with
- 30 Hemacolor. Quantitation of cell movement was the same as for the 30 well chamber. In the inventors' hands, both chemotaxis chambers were equally effective as were both membrane treatments (i.e., with and without a fibronectin coating on the gelatin).

Peptide Synthesis

The synthetic procedures were analogous to those discussed above.

Results

5 The carbon-13 nuclear magnetic resonance (CMR) spectra of the two nonapeptides are give in FIGURE 5, indicating the correctness of synthesis and the purity of the final products. FIGURE 6 shows a phase contrast image of a typical monolayer of bovine aortic
10 endothelial cells, which exhibit a "cobblestone" distribution pattern. After incubation wtih Dil-Ac-LDL, cells showed a punctate fluorescence distribution pattern, indicative of endosomal localization of the labeled LDL derivative internalized by cells via
15 receptor-mediated endocytosis. All cells were positive in fluorescent labeling with respect to controls with variability to cell to cell staining density typically present.

Elastin synthetic peptides are chemoattractants
20 for bovine aortic endothelial cells. FIGURE 7 presents the data collected in an effort to determine the optimal concentration of maximal stimulation of the endothelial cell migration. The dose response curve for the elastin repeat nonapeptide and early passage
25 endothelial cells indicate that maximal activity is a $8 \times 10^{-10}M$ for both peptides, the Phe-containing nonamer (GFGVGAGVP) and the Leu-containing nonamer (GLGVGAGVP). These dose response experiments were repeated 10 times for the Phe-nonapeptide and 9 times
30 for the Leu-nonapeptide; all had the same results

including one study which was counted blind (i.e., the slides were coded and the code was unavailable to the microscopist). FIGURE 7 represents the data from two different experiments. The data were normalized with
5 respect to the fibronectin positive control because of variability in cell responsiveness from experiment to experiment. The left ordinate presents the results as a percent of the fibronectin positive control, the right ordinate gives the actual number of cells
10 migrating per high power field (h.p.f.), for both the Phe and Leu containing nonapeptides. Note the differences in the scales, indicating that in the Phe-nonapeptide experiment, the positive control was 30 cells per h.p.f. and in the Leu-nonapeptide experiment,
15 the positive control was 46 cells per h.p.f. With the data normalized this way, it is evident that both nonapeptides peak at the same concentration and elicit the same degree of responsiveness. The biphasic aspect of the curves may be due to several processes. One is
20 the possibility of saturation of the cell receptors that trigger the chemotactic response and the other is the possibility of gradient breakdown.

Table III presents the data for the checkerboard assay. As expected for chemotaxis, the values along
25 the diagonal indicate zero net cell migration when the concentrations in the two chambers are equivalent, above the diagonal the migration is not significant whereas below the diagonal there is a concentration dependent net movement of cells. These data establish
30 that the repeat elastin nonapeptides promote chemotaxis (i.e., directed movement) rather than solely chemokinesis (i.e., increased random movement). This experiment was repeated 7 times and each experiment had the same pattern.

The hexapeptide of elastin also induced cell migration (FIGURE 8). However, maximal activity shifted by over a decade of concentration to 10^{-8} M. Relative to the fibronectin positive control, the 5 hexapeptide is less active than the two nonapeptides. This experiment was repeated 7 times and the same pattern was obtained.

Table III
Checkboard Analysis of GFGVGAGVP with Endothelial Cells

GFGVGAGVP (M), lower compartment	GFGVGAGVP (M), upper compartment			
	0	10^{-10}	8×10^{-10}	10^{-8}
0	0(16)	4 ± 2	1 ± 2	-3 ± 1
10^{-10}	6 ± 2	1 ± 2	3 ± 2	-1 ± 1
8×10^{-10}	19 ± 3	11 ± 1	-3 ± 1	3 ± 1
10^{-8}	4 ± 2	-1 ± 2	3 ± 2	1 ± 1

Discussion

Peptide purity is an important issue for these chemotactic experiments; one reason is the sharpness of the concentration dependency. If impurities were present from peptide synthesis and varied from lot to lot, inappropriate amounts would be weighed out for the cell migration assay and the peak could easily be missed. Also the impurities themselves could elicit a chemotactic response. The CMR spectra attest to the peptides' purity. As important as a pure chemo-attractant is to the chemotaxis experiment, so too is a pure cell population that responds to a positive control as previous described. FIGURE 6 demonstrates the purity of the cell culture and FIGURE 7 demonstrates that these endothelial cells do migrate towards fibronectin.

The results indicate that the elastin nonapeptides can serve as chemoattractants for endothelial cells. This finding has significance in that the elastin synthetic peptide becomes one of few pure synthetic and chemically defined compounds both to support chemotaxis and to be readily available and at reasonable cost. It also is easily handled, requiring no special storage and dissolution techniques. A wide variety of factors can effect endothelial migration. Some are from blood: platelet factors, lymphocyte products, mitogenic factors from leukocyte cultures, lymphokines, interferon, fibrinogen and its fragments. Many factors have been isolated from tissue in soluble form. These include tumor derived factors, angiogenic factors and preparations and growth factors. There are data indicating that fibronectin alone and/or in concert with gangliosides or heparin constitutes a

chemoattractant for endothelial cells. From this rather extensive listing it is apparent that, aside from fibronectin, fibrinogen and heparin, there are few pure and chemically defined substances that are both
5 supportive of chemotaxis and readily obtainable. The results suggest the use of these nonapeptides as new standards for endothelial cell chemotaxis.

The findings have potential relevance to development of biomaterials, especially in the area of
10 vascular prostheses. Biomaterials that comprise current vascular prostheses do not adequately support the development of an endothelial lining. It is reasonable to suggest that future prostheses will need to use biomaterials that support cell attachment, cell
15 growth and migration of the components of the vascular wall with special emphasis on endothelial cells to improve the patency characteristics of synthetic small vessels. The nonapeptides could provide chemotactic stimuli for endothelial cell migration into the
20 vascular prosthesis.

One of the first morphological events observed in angiogenesis is the mobilization of capillary endothelium. Directed migration of chemotaxis has been proposed as one aspect of the process of angiogenesis
25 and neovascularization. The elastin nonapeptide may be an angiogenic factor in that it promotes directed movement of endothelial cells. It may promote the next step of causing capillaries to infiltrate tissues.

The invention now being fully described, it will
30 be apparent to one of ordinary skill in the art, that many changes and modifications can be made thereto without departing from the spirit or scope of the

invention as set forth herein.

Claims

1. A method of stimulating chemotaxis toward a prosthetic device, which comprises: selecting a chemotactic peptide of the formula



wherein A is a peptide-forming residue of L-alanine;

P is a peptide-forming residue of L-proline;

G is a peptide-forming residue of glycine;

V is a peptide-forming residue of L-valine;

10 F is a peptide-forming residue of

L-phenylalanine;

L is a peptide-forming residue of L-leucine;

B¹ is H or a biocompatible N-terminal group;

15 B² is OH, OB³ where B³ is a non-toxic metal ion, or a biocompatible C-terminal group:

X is GVPGFGVG, GVPGLGVG, VPGFGVG, VPGLGVG, PGFGVG, PGLGVG, GFGVG, GLGVG, FGVG, LGVG, GVG, VG, G or a covalent bond;

20 Y is AGVPGFGV, AGVPGLGV, AGVPGFG, AGVPGLG, AGVPGF, AGVPGL, AGVPG, AGVP, AGV, AG, A or a covalent bond;

n is an integer from 0 to 50;

25 m is an integer from 0 to 50; with the proviso that when both n and m are 0, X and Y are selected so that the chemotactic peptide has at least 3 amino acid residues in the X and Y positions combined; and

30 incorporating said peptide into a layer of a prosthetic device in an amount sufficient to increase the invasion of endothelial cells into said prosthetic device.

2. The method of Claim 1, wherein n is from 1 to 10 and m is 0 to 10.

3. The method of Claim 1, wherein n is about 5 and m is 0 to 5.

4. The method of Claim 1, wherein n is 1 and m is 0.

5. The method of Claim 1, wherein said peptide is H-GFGVGAGVP-OH or a salt thereof.

6. The method of Claim 1, wherein B¹ is H and B² is OH or OB³ where B³ is an alkali metal ion.

7. The method of Claim 1, wherein said amount is from 10⁻⁹ to 10⁻³ millimoles of nonamer or repeating unit per 100 cm² of said surface.

8. The method of Claim 1, wherein said prosthetic device comprises a structural polypeptide.

9. The method of Claim 1, wherein said incorporating involves non-covalent bonding between said chemotactic peptide and said surface.

10. The method of Claim 1, wherein said incorporating involves covalent bonding between said chemotactic peptide and said surface.

11. The method of Claim 10 wherein said surface comprises a structural peptide.

12. A prosthetic device wherein a surface of said device has incorporated into said surface a chemotactic peptide of the formula

B¹-X-(AGVPGLGVG)_n-(AGVPGFGVG)_m-Y-B²
wherein A is a peptide-forming residue of L-alanine;

P is a peptide-forming residue of L-proline;
G is a peptide-forming residue of glycine;
V is a peptide-forming residue of L-valine;
F is a peptide-forming residue of

5 L-phenylalanine;

L is a peptide-forming residue of L-leucine;

B¹ is H or a biocompatible N-terminal group;

B² is OH, OB³ where B³ is a non-toxic metal
ion, or a biocompatible C-terminal group:

10 X is GVPGFVG, GVPGLGVG, VPGFVG, VPGLGVG,
PGFVG, PGLGVG, GFGVG, GLGVG, FGVG, LGVG, GVG, VG, G or
a covalent bond;

Y is AGVPFGV, AGVPGLGV, AGVPFG, AGVPGLG,
AGVPGF, AGVPGL, AGVEG, AGVP, AGV, AG, A or a covalent
15 bond;

n is an integer from 0 to 50;

m is an integer from 0 to 50; with the proviso
that when both n and m are 0, X and Y are selected so
that the chemotactic peptide has at least 3 amino acid
20 residues in the X and Y positions combined.

13. The device of Claim 12, wherein n is from 1
to 10 and m is 0 to 10.

14. The device of Claim 12, wherein n is about 5
and m is 0 to 5.

25 15. The device of Claim 12, wherein n is 1 and m
is 0.

16. The device of Claim 12, wherein said peptide
is H-GFGVGAGVP-OH or a salt thereof.

17. The device of Claim 12, wherein B¹ is H and
30 B² is OH or OB³ where B³ is an alkali metal ion.

18. The device of Claim 12, wherein said amount is from 10^{-9} to 10^{-3} millimoles of nonamer of repeating unit per 100 cm^2 of said surface.

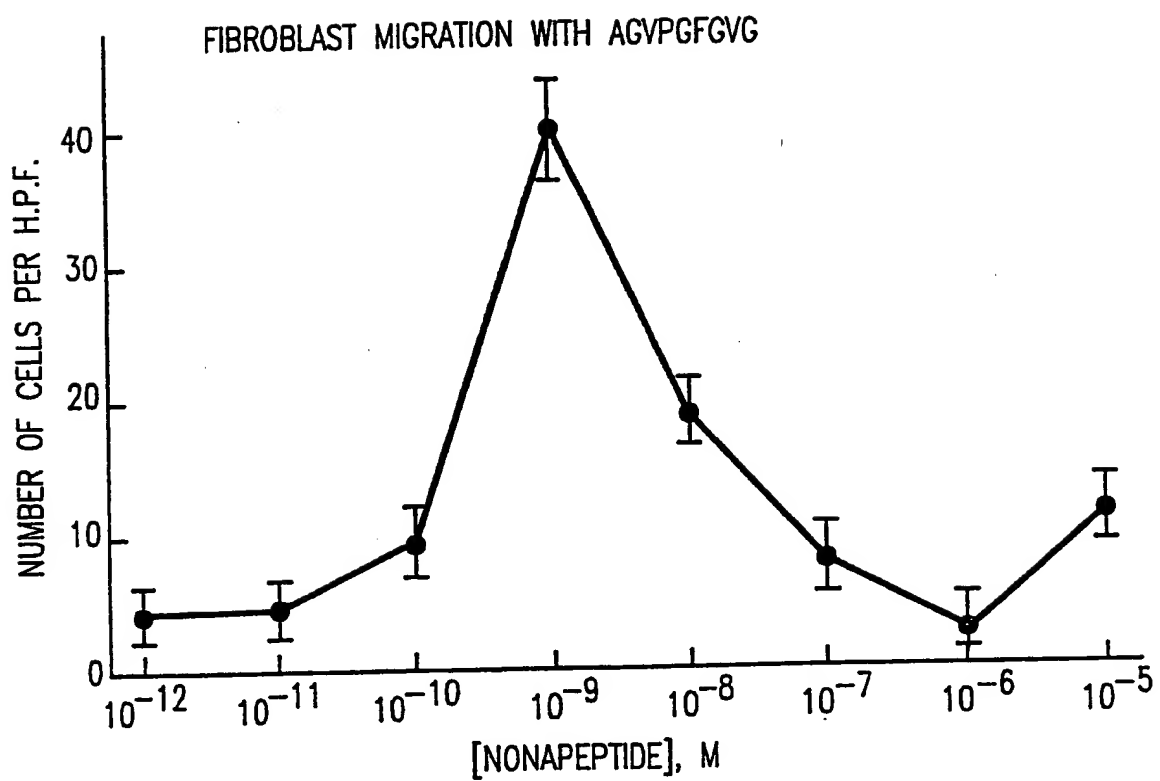
19. The device of Claim 12, wherein said
5 prosthetic device comprises a structural polypeptide.

20. The device of Claim 12, wherein said chemotactic peptide is incorporated using non-covalent bonding between said chemotactic peptide and said surface.

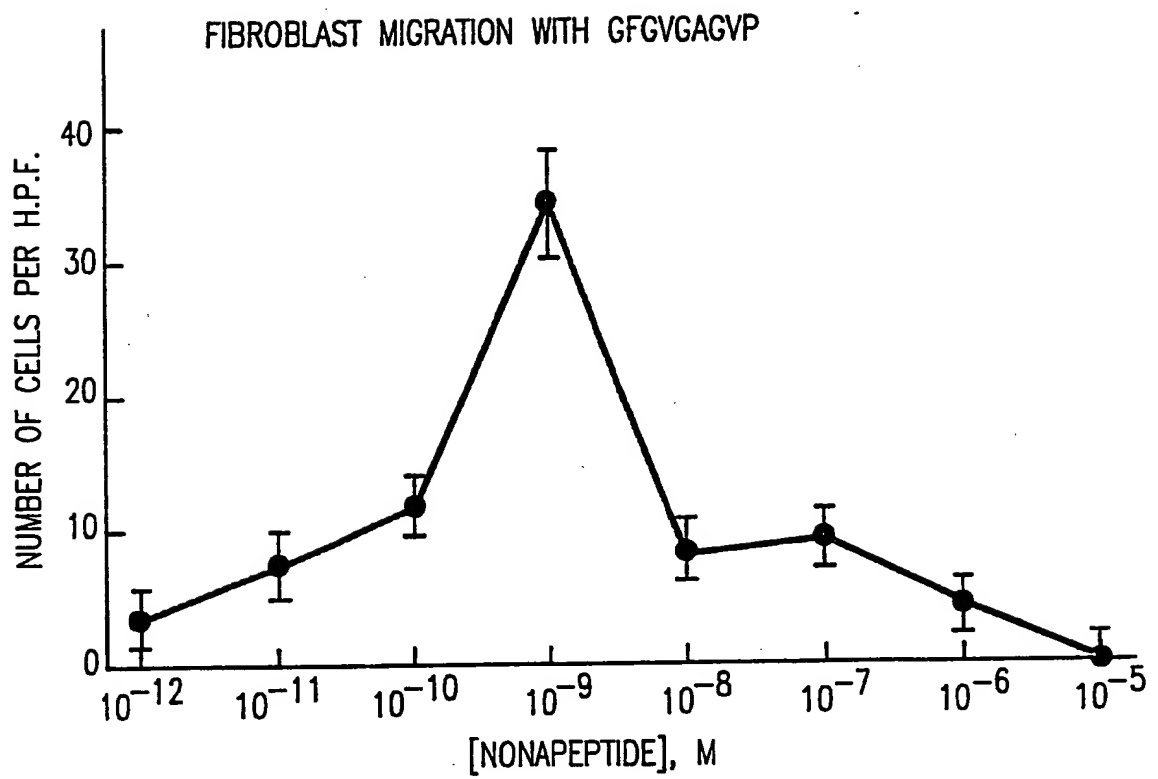
10 21. The method of Claim 12, wherein said chemotactic peptide is incorporated using covalent bonding between said chemotactic peptide and said surface.

22. The device of Claim 21, wherein said surface
15 comprises a structural peptide.

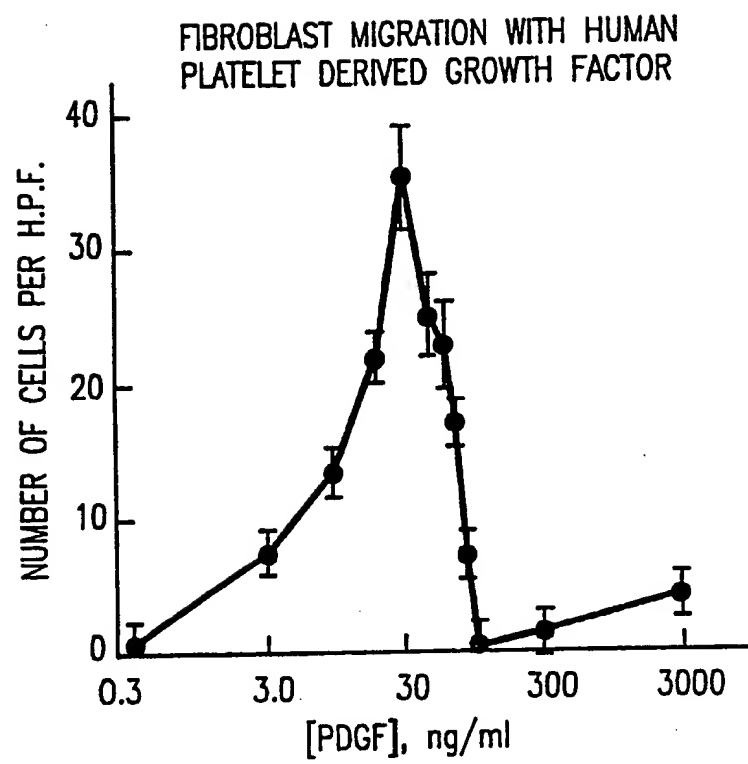
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**FIG. 1**

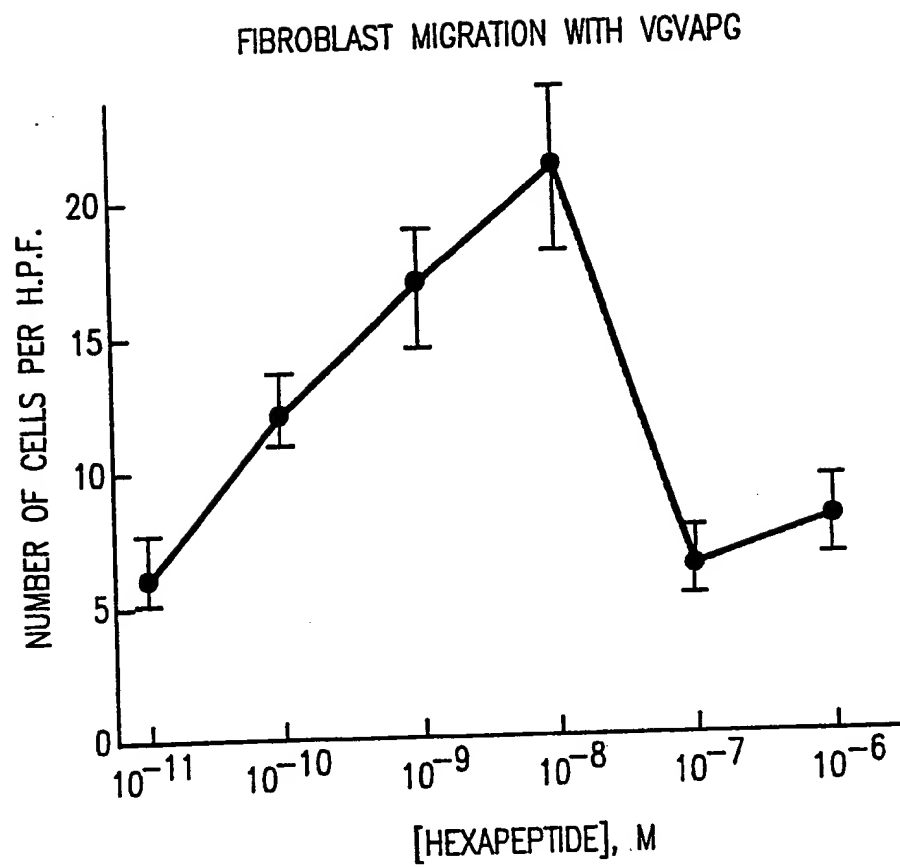
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**FIG. 2**

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**FIG. 3**

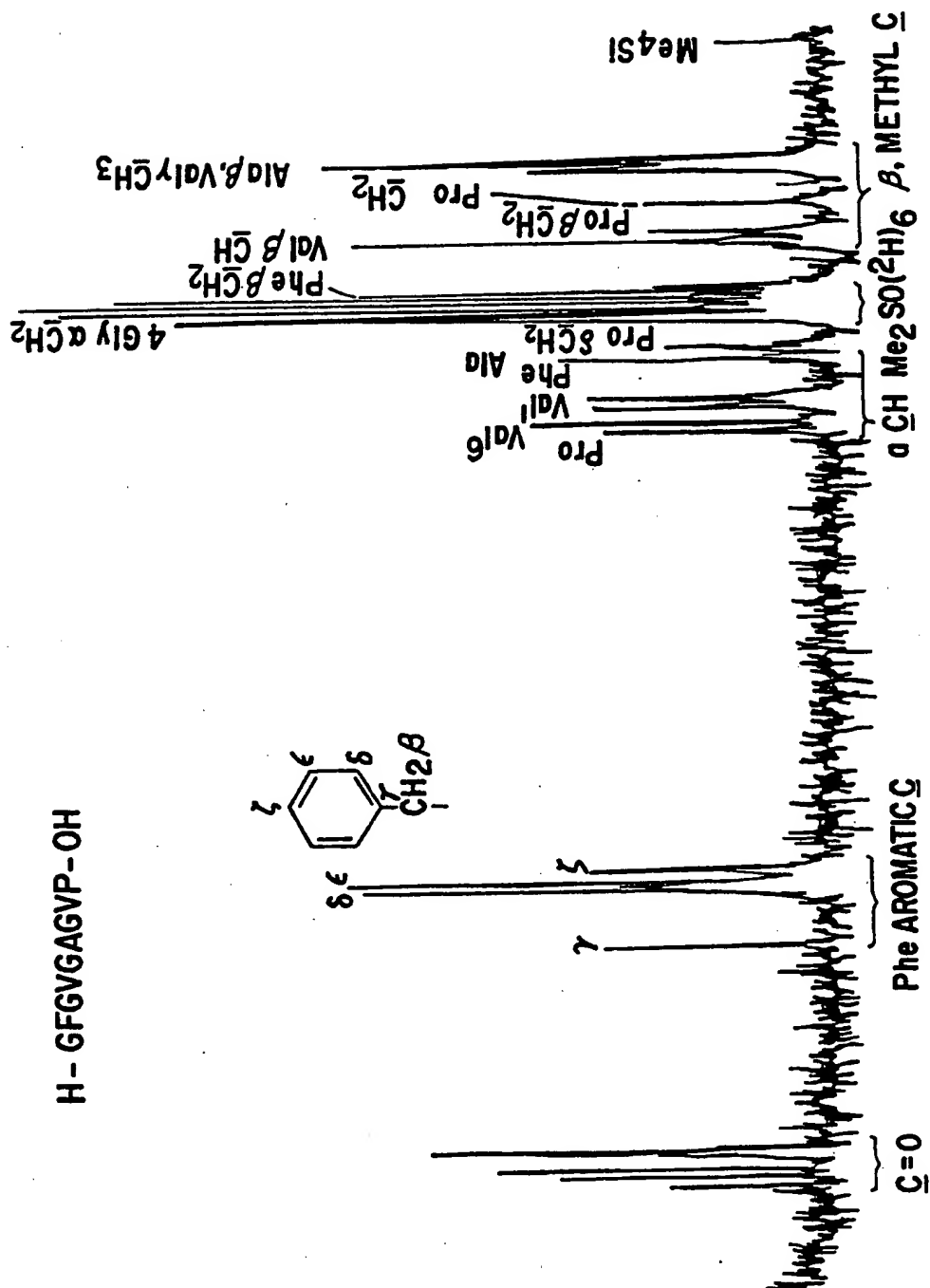
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**FIG. 4**

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FIG. 5A

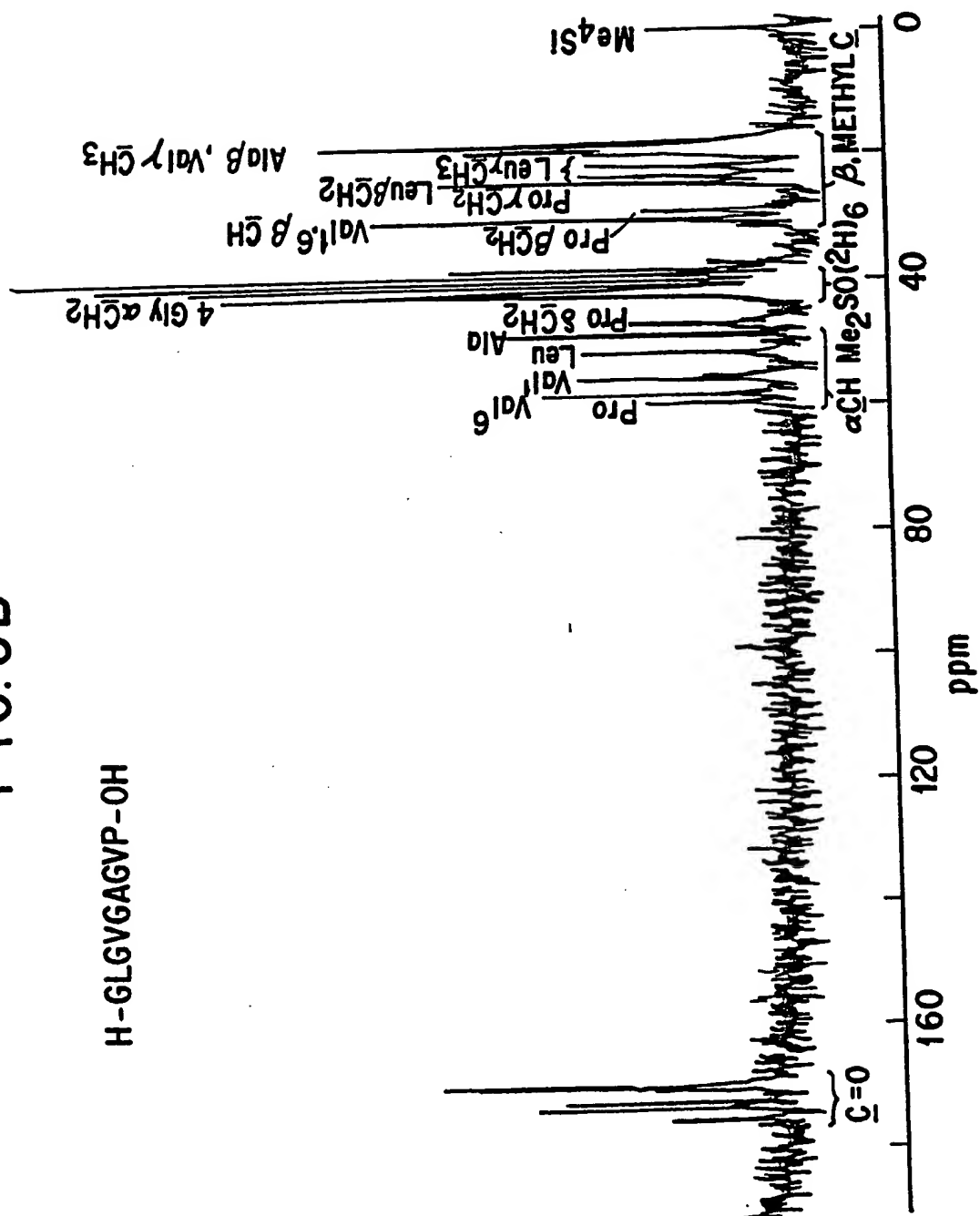
H - GFGVGAGVP - OH



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FIG. 5B

H-GLGVGAGVP-OH

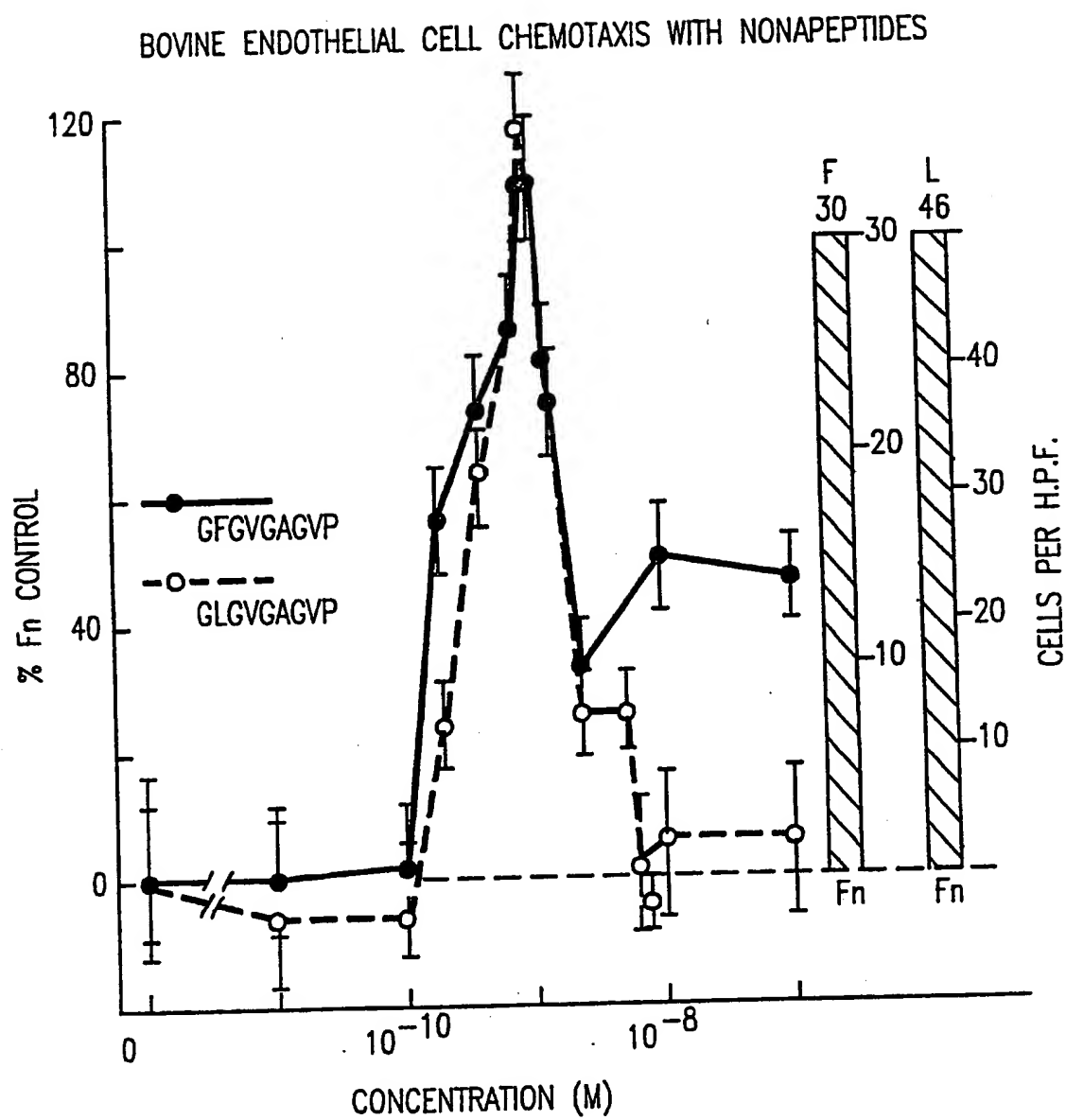


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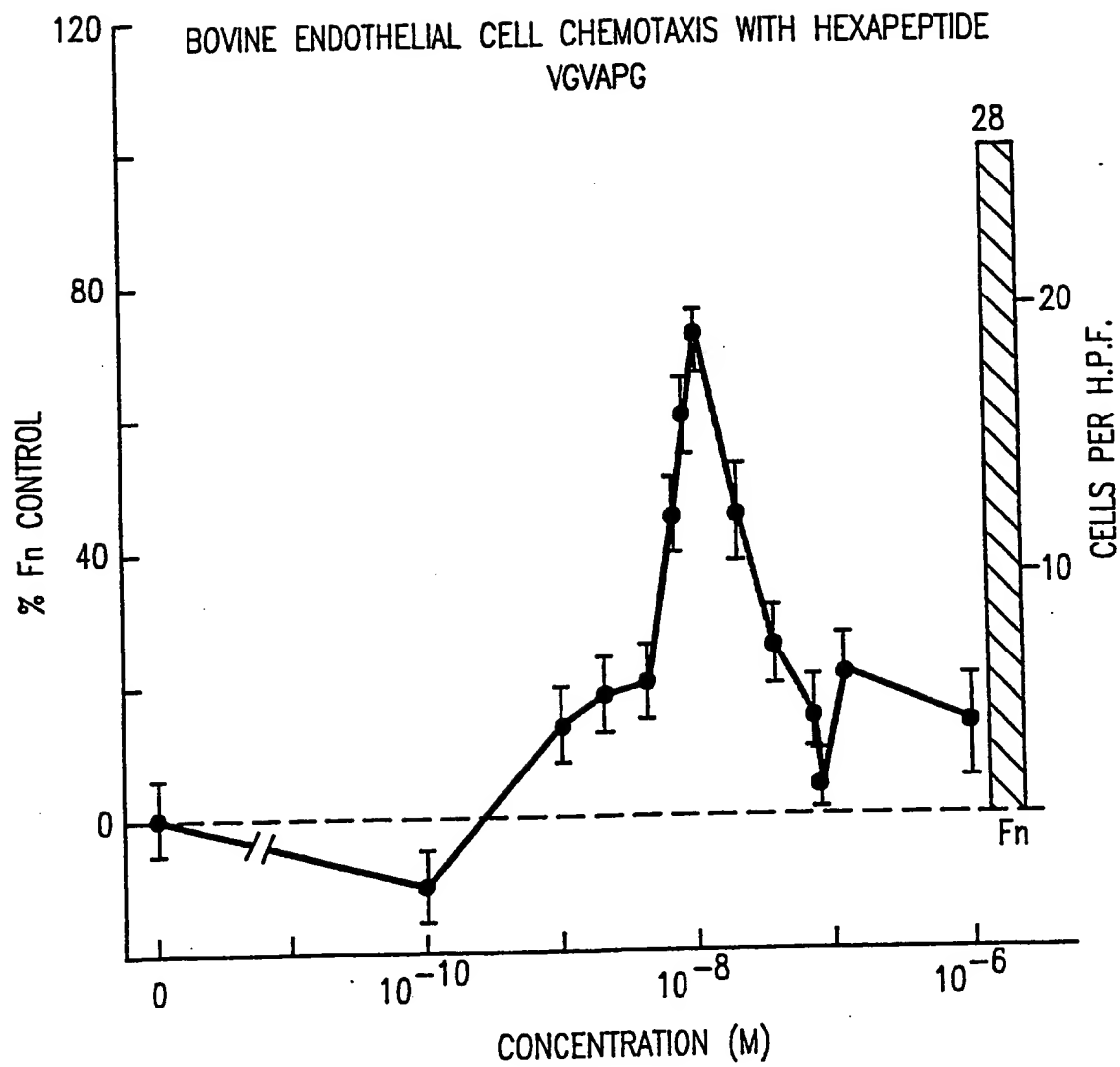
FIG. 6



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**FIG. 7**

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**FIG. 8**

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/01321

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC (4): A61F 2/02 U.S. Cl. 623/11, 66																	
II. FIELDS SEARCHED <div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched ⁷</div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%;">Classification System</th> <th>Classification Symbols</th> </tr> <tr> <td style="text-align: center; vertical-align: top;">U.S.</td> <td>623/11, 1, 66; 530/328, 353; 514/15; 427/2</td> </tr> </table> <div style="text-align: center; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	U.S.	623/11, 1, 66; 530/328, 353; 514/15; 427/2											
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%;">Category ¹⁰</th> <th style="width: 70%;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td>US, A, 4,605,413 (URRY ET AL.) 12 August 1986, see the entire document.</td> <td style="text-align: center; vertical-align: top;">1-11</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td>US, A, 4,578,079 (RUOSLAHTI ET AL.) 25 March 1986, see column 6, lines 49-60 and column 7, lines 1-4.</td> <td style="text-align: center; vertical-align: top;">1-6, 8, 10, 11</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td>US, A, 4,693,718 (URRY ET AL.) 15 September 1987, see column 13, line 26-column 4, line 32.</td> <td style="text-align: center; vertical-align: top;">12-22</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td>US, A, 4,589,881 (PIERSCHBACHER ET AL.) 20 May 1986, see column 1, lines 30-50.</td> <td style="text-align: center; vertical-align: top;">12-22</td> </tr> </table>			Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	US, A, 4,605,413 (URRY ET AL.) 12 August 1986, see the entire document.	1-11	X	US, A, 4,578,079 (RUOSLAHTI ET AL.) 25 March 1986, see column 6, lines 49-60 and column 7, lines 1-4.	1-6, 8, 10, 11	A	US, A, 4,693,718 (URRY ET AL.) 15 September 1987, see column 13, line 26-column 4, line 32.	12-22	A	US, A, 4,589,881 (PIERSCHBACHER ET AL.) 20 May 1986, see column 1, lines 30-50.	12-22
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;"> Date of the Actual Completion of the International Search <div style="text-align: center; font-size: 1.2em;">07 JULY 1989</div> </td> <td style="width: 50%; padding: 5px;"> Date of Mailing of this International Search Report <div style="text-align: center; font-size: 1.2em;">26 JUL 1989</div> </td> </tr> <tr> <td style="width: 50%; padding: 5px;"> International Searching Authority <div style="text-align: center;">ISA/US</div> </td> <td style="width: 50%; padding: 5px;"> Signature of Authorized Officer <div style="text-align: center;">A. Cannon </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center; font-size: 1.2em;">07 JULY 1989</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-size: 1.2em;">26 JUL 1989</div>	International Searching Authority <div style="text-align: center;">ISA/US</div>	Signature of Authorized Officer <div style="text-align: center;">A. Cannon </div>											
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